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## EXPERIMENTS ON THE DEVELOPMENT OF EGG FRAGMENTS IN CEREBRATULUS.<sup>1</sup>

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The question, "To what extent can the principle of germinal localization be applied to the unsegmented egg, and how far, on the other hand, may the specification of the embryonic regions be considered a progressive process that falls under the category of epigenetic phenomena?" (Wilson, 1903<sup>2</sup>) led me in the summer of 1903 to carry out a series of removal-experiments on the eggs of *Cerebratulus lacteus*, during the later part of my stay at the Tufts College Marine Laboratory at Harpswell, Me. I wished to determine whether the cytoplasmic localization is progressively established, and especially to ascertain the conditions existing in the egg just prior to the first cleavage. To this end I have examined the development of egg fragments, obtained by cutting, at four successive periods between discharge of the egg and the first cleavage, namely, (1) before the dissolution of the germinal vesicle, (2) at the metaphase of the first polar mitosis, (3) at the period of conjugation of the egg- and sperm-nuclei and (4) after the constriction of the first cleavage appeared. The result shows that the percentage of abnormal larvæ steadily increases as the egg approaches the two-cell stage. When I appreciated the importance of comparing with these results the development of isolated blastomeres of the two-cell stage, the breeding season was nearly over, and I was only able to make a few experiments, which are not numerous enough to give a satisfactory basis of comparison. I hope, however, to carry on more complete experiments at the first opportunity.

A word about cutting the eggs. A drop of water containing several eggs was spread on a slide, and some of the water was

<sup>1</sup> For the preparation of the present paper my best thanks are due to Professor E. B. Wilson for his kindly suggestions and criticisms. I am also indebted to Professor J. S. Kingsley for kindness shown me at the Harpswell Laboratory.

<sup>2</sup> E. B. Wilson, "Experiments on Cleavage and Localization in the Nemertine Egg," *Arch. f. Entwum.*, Bd. 16, Heft 3, p. 440.

removed by a pipette. The egg, thus flattened a little, was cut with a fine scalpel. It sometimes proved better, in order to spread the water evenly, to use a little albumen fixative, rubbed on the slide, and thoroughly washed off with a brush. In doing so, the quantity of glycerine left on the slide was infinitesimal, so that it did not affect at all the development of the eggs. My experiments consisted in cutting off a portion of cytoplasm from unsegmented eggs at the four periods already mentioned, in the first two cases fertilizing the fragments, and rearing the resulting embryos up to the pilidium. For the sake of uniformity I have confined my work to the nucleated fragments. Cleavage was studied up to the eight-cell stage. Among the pilidia thus obtained, I found many abnormal ones, and they were compared with the normal larvæ. Every pilidium was drawn with a camera between 48 and 58 hours after fertilization. During drawing they were kept still by sucking out some of the water from under the cover-glass, which was supported by a piece of thread. Thus they could barely move without distortion of their shape. It should here be noted that defective larvæ remain always defective; moreover, the defective parts become more and more prominent as the larvæ grow. No size regulation takes place among the pilidia; the smaller the original egg pieces, the smaller the pilidia.

#### SERIES A.

##### *Development of Fragments obtained before the Disappearance of the Germinal Vesicle.*

A portion of cytoplasm was cut off from the egg immediately after its release and while the germinal vesicle was intact, and the nucleated piece was fertilized. Care was taken not to injure the germinal vesicle, and most of the operations were done upon eggs in which it was eccentrically situated, so as to cut off as much cytoplasm as possible. Since the germinal vesicle, as a rule, lies nearer to the animal pole, it may be inferred that most of my sections were performed in the vegetative hemisphere, although I did not record the exact plane in this series.

Thirty-five egg-fragments were able to develop up to the pilidium stage, the rest having either died or been rejected on account of polyspermy. The result may be tabulated as follows:

Perfect pilidia 30 { smaller 6 larger 24 }	.....	85.7 per cent.
Defective pilidia 5	.....	14.3 per cent.

It is striking that comparatively few larvæ turned out to be defective. All of these are shown in Fig. 1, *C-G*,<sup>1</sup> a glance at which shows that they are certainly abnormal, yet their defect is not so great as those obtained at later periods (Series B and C).

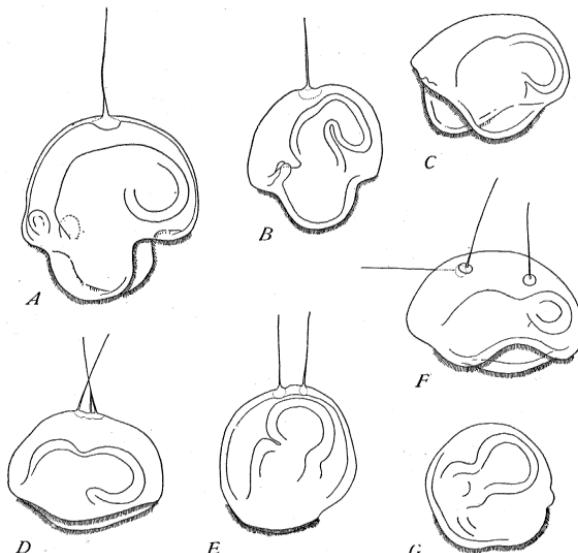


FIG. I.

*1C* represents a larva which would be perfect, if there were an apical organ. *1D* is another defective one with three apical organs, two tufts having been about to fuse; in other respects it is perfect. A pilidium represented in *1E* is much more abnormal than the preceding two. It has two apical organs and a pair of ciliated lappets of smaller size. The shape of the gut also is not normal. Another pilidium (*1F*) resembles the one with three apical organs just described (*1D*), but differs from it in having three apical organs widely separated from one another—two on the left side, one on the right. In this larva the left ciliated lobe is indented. Lastly we have a very defective pilidium (*1G*), which, like *1C*, has no apical organ. While the ciliated lobes

<sup>1</sup>All the figures throughout the paper have been drawn by the camera; 84 diameters magnification.

are comparatively normal, the gut is quite abnormal, the stomach being larger than the oesophagus, and the position of the mouth deviating from the normal. Nevertheless each part is complete, except the apical organ. It should not be overlooked that the apical organ is abnormal in every one of five defective pilidia, while both the gut and ciliated lobes are fairly unaffected by the operation. From this it is probable that the basis of the apical organ is vaguely foreshadowed as early as the stage at which the germinal vesicle is still intact.

It is highly important to note that the formation of a pilidium does not depend on the size of the piece, since one fourth of the perfect larvæ are decidedly smaller than the rest. The larva, 1A, is one of the larger group, and 1B one of the smaller. Both are perfect in every respect, but one is a little larger than one half of the other. From the fact that, in spite of cutting the eggs at random, a large percentage of perfect larvæ were produced, that the perfect ones vary greatly in size, and that the defects are not so considerable as at a later period, the most natural interpretation one can draw would be that, antecedent to the dissolution of the germinal vesicle, the egg cytoplasm still shows little or no definite specification of the germ regions.

#### SERIES B.

##### *Development of Fragments obtained at the Metaphase of the first Polar Mitosis.*

The eggs were cut when the mitotic figure of the first maturation division was completed; this can readily be seen as a clear space at the animal pole. The nucleated fragment was fertilized. Sixty-five eggs thus operated were able to develop into larvæ. Owing to a change of consistency of either the gelatinous egg envelope or the egg itself, I found it more difficult to operate at this stage than at any other. The result was :

Perfect pilidia 34.....	52.3 per cent.
Defective pilidia 31.....	47.7 per cent.

Notwithstanding the difficulty of the operation, it was comparatively easy to cut off a considerable part of cytoplasm from the egg of this stage on account of the peripheral position of the

mitotic figure. Consequently in some cases the fragments were quite small. In this series all the defective larvæ were more abnormal than in the preceding. The abnormalities may be classified as follows:

Defect in apical organ.	{ Larvæ with supernumerary apical organ .....	4	14
	" " no apical organ .....	10	
Defect in ciliated lobes.	" " ciliated lobes indented or not well developed	16	27
	" " only one ciliated lobe .....	11	
	" " no gut.....	6	21
	" " no oesophagus .....	5	
Defect in gut.	" " no stomach.....	1	21
	" " stomach cut off from oesophagus.....	2	
	" " gut not well developed.....	7	

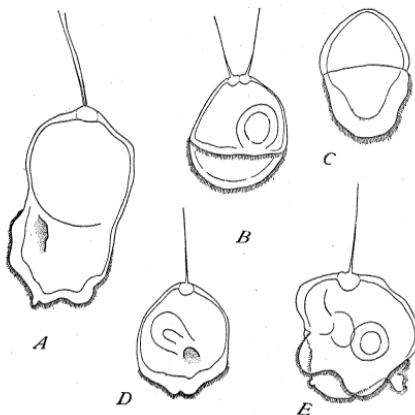


FIG. 2.

The disturbance of the apical organ ( $2B$  and  $2C$ ) is of special interest, indicating that its basis covers quite a large area over the animal pole, since in this series it was impossible to cut off cytoplasm from the top of the egg. While in Series A the defective part was chiefly restricted to the apical organ, we find that in the stage under consideration the abnormalities extend to the ciliated lobes and gut also. As will be seen from the above table, a large number of the defective pilidia have abnormal ciliated lobes; in some, one of the lobes is very short ( $2B$ ), while in others the lobes are indented ( $2E$ ), reminding us of *Pilidium branchiatum*. Quite commonly one lobe is entirely wanting ( $2A$ ,  $2C$  and  $2D$ ). The defect in the gut is remarkable. In a few larvæ

there is no gut at all, these being not far from the "Dauerblas-tulæ" (2C); in other cases the gut is represented by a shallow depression (2A). In some defective larvæ either the stomach or the oesophagus may be wanting (2B, 2D). In a few cases the stomach is cut off from the oesophagus (2E).

It is noteworthy that there occur a few defective larvæ which are as large as the perfect ones (2A). And among the perfect pilidia only three were smaller than the others.

From the increased number of defective larvæ in this series, it may be inferred that, since the fading of the germinal vesicle, the regional specification has advanced. But the occurrence of perfect pilidia equal in number to the defective ones cannot be overlooked. It is possible that the production of the perfect larvæ is because the injury was too small to have materially affected the organ-bases, or because the plane of section was such as not to disturb their normal proportions. The number obtained is, however, too great to lend much probability to either of these suggestions. A more likely interpretation is that the egg still possesses a considerable power of regulation.

#### SERIES C.

##### *Development of Fragments obtained at the Period of Conjugation of the Egg- and Sperm-nuclei.*

The eggs were fertilized and a portion of cytoplasm was cut off a little after the second polar body was extruded, *i. e.*, at about

the stage at which the egg and sperm nuclei came to fuse. In this series the polar bodies gave a very good landmark for orientation of the egg. The accompanying cut shows the direction of section plane (Fig. 3). The eggs which were able to develop up to pilidium were not as numerous as in Series B, but they gave a fairly conclusive result, as shown in the table on p. 102.

Most of the pilidia of this series are defective, as tabulated. Not only that, the defective parts correspond in a general way to the region from which the cytoplasm was cut.<sup>1</sup> It is very

<sup>1</sup> The apparent contradictory result as in the case of Nos. 17 and 23 is probably due to the volume of the cytoplasm cut off.

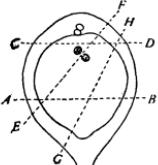


FIG. 3.

No. of Eggs.	Section Plane.	Condition.	Remarks.
1	AB	D (effective)	Ciliated lobes short.
2	"	D	No apical organ; ciliated lobes asymmetrical.
3	"	D	One of ciliated lobes wanting.
4	"	D	Gut not well developed.
5	"	P (perfect)	Apical organ on the right side.
6	CD	P	
7	"	P	
8	"	P	
9	"	P	
10 <sup>1</sup>	"	D	Two apical organs. Gut double.
11	EF	D	Ciliated lobes defective.
12	"	D	One of ciliated lobes wanting.
13	"	D	One of ciliated lobes defective.
{ 14	"	D	Gut defective.
{ 14'	"	D	No apical organ; one ciliated lobe wanting; gut comparatively large.
{ 15	"	D	One of ciliated lobes wanting.
{ 15'	"	D	One of ciliated lobes wanting.
16	GH	P	
17	"	D	One of ciliated lobes wanting.
18	"	D	Neither of ciliated lobes developed, but the margin ciliated.
19	"	D	One of ciliated lobes wanting.
20	"	D	Ciliated lobes defective.
21	"	D	Ciliated lobes defective.
22	"	D	One of ciliated lobes wanting.
{ 23 <sup>2</sup>	"	D	No apical organ; only one lobe present; gut imperfect.
{ 23'	"	D	No apical organ; only one lobe present; gut imperfect.

## Summary :

Perfect pilidia	6.....	24 %
Defective pilidia	20.....	76 %

important to observe that most of the perfect larvæ were produced from the egg cut along *CD*. The basis of the apical organ must, therefore, have existed below the line *CD*. This fact, and the frequent occurrence of a defective apical organ in series *B* taken together, it may safely be concluded that the basis of this organ takes the form of a broad ring a little above the equator. But how it finally takes an apical position I am at a loss to imagine. Although Professor Wilson's experiments<sup>3</sup> were not directed to this problem, his results seem to tally well with

<sup>1</sup> In this egg the nuclei conjugated in the vegetative hemisphere; the section plane passed nearly through the equator.

<sup>2</sup> In this egg the sperm-nucleus must have been present in the piece which I thought enucleated.

<sup>3</sup> Wilson, *I. e.*, pp. 432, 433 (Fig. 10), 436.

my conclusion. He obtained through the section near the animal pole along  $\phi\phi$  (his Fig. 3, *B*, on p. 240) the animal larva without any apical organ, the vegetative one in this case having been provided with the multiple apical organs (his Fig. 10, *E*, on p. 433). On the other hand, in another case, he found that both the animal and vegetative larvæ had the normal apical organ

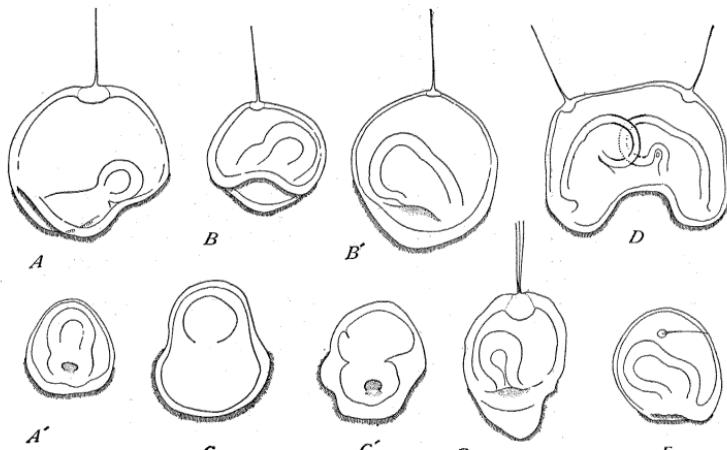


FIG. 4.

(his Fig. 10, *A* and *B*) when he cut along the plane near the equator ( $kl$  of his Fig. 3, *B*). In this case it may be inferred that the section plane bisected the basis of the apical organ. He gives still another instance of the animal larva with the apical organ (his Fig. 10, *C*) obtained when he cut below the equator along  $mm$  (his Fig. 3, *B*).

The pilidium No. 10 (4*D*) is a very important one. It was produced from an egg from which about one third of the animal hemisphere was cut off. This was about as large as a perfect larva if seen from the side, but it was extremely compressed laterally. It has not only two apical organs but two guts. I suspect that it might have been disturbed by some unknown cause at the two-cell stage because, as a rule, half-larvæ obtained by isolation of blastomeres are larger than one half of the normal larvæ. In another pilidium (No. 4) I found the apical organ shifted from the normal position (4*F*). In spite of this, it swam with the apical pole directed forward like a normal pilidium.

In three cases I got two larvæ from one fertilized egg. There are three possibilities to explain this: first, when the operation is done before the fusion of the egg- and sperm-nuclei; second, after the first cleavage mitosis came to the telophase; third, when the egg is doubly fertilized and the segmentation nucleus is cut apart from the sperm-nucleus. Since my operations were done not so late as the second case, the result may be due either to the first or the third cause. Whatever the cause may be, the comparison of the resulting larvæ is very interesting.  $4A$  and  $4A'$  (Nos. 14 and 14') show a most instructive pair of pilidia. The larger of the two is almost normal, except that the gut is very defective, while the smaller one has a comparatively large gut. Professor Wilson found a similar pair of larvæ by cutting the blastulas (cf. his Fig. 11, *A* and *B*). Another pair ( $4B$  and  $4B'$ , Nos. 15 and 15') are also important; both of them are normal, but one ciliated lobe is wanting in each. In still another pair,  $4C$  and  $4C'$  (Nos. 23 and 23'), both are devoid of the apical organ and have a very defective gut. Either of them is barely more than the lappet.

Now let us see how the basis of the ciliated lobes and gut are disposed in the egg of this stage. Most of the defective larvæ have abnormalities in the ciliated lobes in some way or other. We can distinguish two kinds of defect; in one only one ciliated lobe has been developed, the other being entirely suppressed, so that the mouth can be seen from the side ( $4A'$ ,  $4B'$ ,  $4C'$  and  $4E$ ). The other kind of defect is shown in  $4F$ . In this case both the ciliated lobes are present, but they are very short and almost straight. This difference may be ascribed to the fact that the basis of the ciliated lobes is more or less bilaterally situated in the vegetative hemisphere near the equator. As for the basis of the gut I know very little, but it is certain that it lies near the negative pole (cf.  $4A$  and  $4A'$ ).

#### SERIES D.

##### *Development of Fragments obtained before the Completion of the first Cleavage.*

The eggs were cut between the period of the appearance of the first cleavage furrow and the completion of the division. This

experiment was begun at the end of my stay at Harpswell, and I can give here only four cases.

(1) The egg was cut along *AB* (5*A*) and a perfect pilidium was produced which is represented in 5*C*. It is important to observe that, in spite of cutting off the cytoplasm from the animal pole, the apical organ has developed undisturbed.

(2 and 3) The eggs were cut along *CD* (5*A*). From each blastomere arose a pilidium with one ciliated lobe and no apical organ. The cases are too few to draw any conclusion, but the importance of the cytoplasmic bridge connecting the blastomeres and of constriction of the first cleavage upon the arrangement of organ bases, is not to be overlooked.

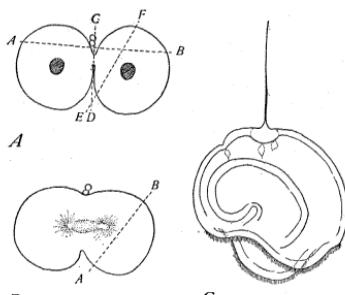


FIG. 5.

(4) The egg was cut along *EF* (5*A*). From the left half a perfect but dwarf pilidium resulted. This is a case worth describing, because the egg neither rounded up, as is usually the case, nor divided into two unequal halves, but soon after the cut surface was closed, the right half gradually increased in size accompanied by the decrease of the left half, and thus the egg was divided into two equal blastomeres.

#### SERIES E.

##### *Development of the Blastomeres Isolated at the Two-cell Stage.*

The blastomeres of eight eggs were separated at the two-cell stage when the cytoplasmic bridge had disappeared and the blastomeres had assumed a spherical form. From every blastomere a normal pilidium was developed. Some were about half the normal size, while some, for reasons that I cannot explain, were very

much smaller. It is remarkable that the development of the isolated blastomeres differs very widely from that of fragments of the period preceding. From a comparison with Series D it seems likely that the result may be different according to the period of cutting — *i. e.*, before or after the disappearance of the cytoplasmic bridge (cf. Series D, eggs *B* and *C*) and the pressing together of the blastomeres. In any event, further careful examination of the egg of this stage with reference to the question of egg specification is very desirable.

#### CONCLUSION.

From the above series of experiments we have seen that, if the cutting-off of a portion of cytoplasm is done before maturation, it only slightly affects the normal development; but if the operation is done at the first maturation stage the formation of the organs of the pilidia is considerably affected, and still more if it is done at the time of fusion of the germ-nuclei. We cannot, therefore, escape the conclusion that there must take place some progressive changes in the general make-up of the egg during the period extending from the time of dissolution of the germinal vesicle to the fusion of the germ nuclei. This period falls into two subdivisions, the first extending from the fading of the germinal vesicle to the metaphase of the first polar mitosis, and the second from the growing period of the sperm-nucleus to its conjugation with the egg-nucleus. The importance of these two periods has been correctly perceived by Delage (1901), Fischel (1903) and recently by Professor Wilson (1903). It is hardly necessary to state here that, although these two periods can be artificially separated by delaying fertilization, yet, under natural conditions, there is no pause between them, as the spermatozoon enters the egg before its maturation.

Let us next see what visible changes take place in the egg cytoplasm as the result of the above two acts, *i. e.*, the disappearance of the germinal vesicle and the entrance of the spermatozoon in the matured cytoplasm. As soon as the germinal vesicle breaks up, the nuclear fluids flow out and may diffuse through the egg. At this time currents may be formed carrying the egg cytoplasm from one spot to another. Although

in the *Cerebratulus* egg we have so far no direct evidence to support the occurrence of the last phenomenon, yet my observations on sections clearly show that a segregation of egg material does actually takes place at this period, the yolk accumulating in the lower hemisphere, while the clear and more finely granular protoplasm collects especially at the top of the egg, where, in the iron hematoxylin-Congo-red preparations, it stands out beautifully stained blue in contrast with the red yolk. After the fading of the germinal vesicle the eggs not only become fecundable, but also acquire much more power of forming cytasters than they had before. It is hardly necessary to state that in many forms remarkable changes take place in the egg at or after the entrance of the spermatozoon. It may further be pointed out, however, that the part played by the spermatozoon in causing rearrangements of the egg-substance is of a subsidiary character, as is shown in the case of parthenogenesis.

To the question as to what degree of localization exists in the cytoplasm of the *Cerebratulus* egg before the fading of the germinal vesicle, I am not in a position to answer, and it is almost impossible to find direct evidence. But the results of my experiments harmonize with Boveri's view that there is at first only a simple promorphological condition such as polarity and bilaterality, which may give a basis, so to speak, for a more definite grouping of material arising at the time of the flowing-out of the nuclear fluids. Otherwise it is impossible to understand the sudden increase in the proportion of abnormal embryos arising from the fragments obtained subsequent to the fading of the germinal vesicle. In the eggs of some forms (*e. g.* *Myzostoma*) the segregation of material has in a measure taken place long before maturation. In such cases the horizontal section of the egg before the disappearance of the germinal vesicle will, I think, produce a defective embryo. My observations on the later periods render it probable that at this time there must be a certain number of predetermined regions more or less firmly fixed, though this is not so clearly shown as in the ctenophore egg. It is extremely desirable to carry out careful studies with respect to the egg specification prior to the first cleavage in other forms.

So far as I could ascertain (up to the eight-cell stage), the cleav-

age of egg fragments was perfectly normal in Series A, B and C. It is especially noteworthy that the nucleated pieces of the fertilized eggs cleave exactly like a normal egg. Whatever amount of cytoplasm be cut off, the nucleated piece always divides at first into two and then into four equal blastomeres. Since these fragments so often gave rise to defective embryos, it is probable, not only that the two and four blastomeres differ from each other, but also that they do not correspond with those in the normal case. The same is true in the eight-cell stage, and so on. From this it seems further probable that cleavage of an egg takes its normal course irrespective of the localization of embryonic regions within. The cleavage pattern is stamped on the cytoplasm; but the end-result is governed by a different set of factors. The cleavage factors seem, therefore, to differ in this case from the morphogenic ones. "Though there is often a close and constant connection in the normal development between the process of cleavage and that of localization and differentiation, this connection is not a necessary relation" (Wilson, 1903<sup>1</sup>).

It is rather striking that, if the operation is done before the completion of the first cleavage, most of the egg fragments give rise to defective larvæ, while all of the isolated blastomeres at the two-cell stage develop into perfect pilidia (though the result is not conclusive, since my cases were too few). It is easy to conceive, however, that natural cleavage comes into operation in a quite different way from the artificial section, and it is probable that by the natural cleavage all the organ bases are equally distributed into two blastomeres which would be very unlikely after an artificial section.

#### SUMMARY.

1. Before the germinal vesicle fades, there is no evidence of definite specification in the egg regions.
2. Dissolution of the germinal vesicle initiates the establishment of the germinal localization.
3. In the period between the entrance of the spermatozoon and the fusion of the germ nuclei, the localization becomes more definite.
4. The basis of the apical organ is not at the animal pole, but

<sup>1</sup> Wilson, *I. c.*, p. 439.

somewhere above the equator as a broad zone. The bases of the ciliated lobes and the gut lie mainly in the vegetative hemisphere.

5. The cleavage (up to eight-cell stage) is normal in an egg fragment obtained from the unsegmented egg, whatever be the amount of cytoplasm cut off, or at whatever period. It is probable that the cleavage factors do not here necessarily coincide with the morphogenic ones.

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October 29, 1903.